

AB

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
20 March 2003 (20.03.2003)

PCT

(10) International Publication Number
WO 03/023004 A2

(51) International Patent Classification⁷: C12N (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(21) International Application Number: PCT/US02/28549

(22) International Filing Date: 6 September 2002 (06.09.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 09/953,047 10 September 2001 (10.09.2001) US

(71) Applicant: **ISIS PHARMACEUTICALS, INC.** [US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008 (US).

(72) Inventors: **MONIA, Brett, P.**; 7605 Nueva Castilla Way, La Costa, CA 92009 (US). **WYATT, Jacqueline, R.**; 1065 Hymetus Avenue, Encinitas, CA 92024 (US).

(74) Agents: **LICATA, Jane, Massey et al.**; Licata & Tyrrell P.C., 66 E. Main Street, Marlton, NJ 08053 (US).



Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/023004 A2

(54) Title: ANTISENSE MODULATION OF FIBROBLAST GROWTH FACTOR RECEPTOR 3 EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of fibroblast growth factor receptor 3. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding fibroblast growth factor receptor 3. Methods of using these compounds for modulation of fibroblast growth factor receptor 3 expression and for treatment of diseases associated with expression of fibroblast growth factor receptor 3 are provided.

5 **ANTISENSE MODULATION OF FIBROBLAST GROWTH FACTOR RECEPTOR 3
EXPRESSION**

10 **FIELD OF THE INVENTION**

The present invention provides compositions and methods for modulating the expression of fibroblast growth factor receptor 3. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with 15 nucleic acids encoding fibroblast growth factor receptor 3. Such compounds have been shown to modulate the expression of fibroblast growth factor receptor 3.

20 **BACKGROUND OF THE INVENTION**

The fibroblast growth factor (FGF) family of signaling polypeptides regulates a diverse array of physiologic functions including mitogenesis, wound healing, cell differentiation and angiogenesis, and development. Both normal and malignant cell growth as well as proliferation are affected by changes in local 25 concentration of these extracellular signaling molecules, which act as autocrine as well as paracrine factors. Autocrine FGF signaling may be particularly important in the progression of steroid hormone-dependent cancers and to a hormone independent state (Powers et al., *Endocr. Relat. Cancer*, 2000, 7, 165-197). 30 FGFs and their receptors are expressed at increased levels in several tissues and cell lines and overexpression is believed to contribute to the malignant phenotype. Furthermore, a number of oncogenes are homologues of genes encoding growth factor receptors, and there is a potential for aberrant activation of 35 FGF-dependent signaling in human pancreatic cancer (Ozawa et al., *Teratog. Carcinog. Mutagen.*, 2001, 21, 27-44).

The two prototypic members are acidic fibroblast growth factor (aFGF or FGF1) and basic fibroblast growth factors (bFGF

-2-

or FGF2), and to date, at least twenty distinct FGF family members have been identified. The cellular response to FGFs is transmitted via four types of high affinity transmembrane tyrosine-kinase fibroblast growth factor receptors numbered 1 to 5 4 (FGFR-1 to FGFR-4). Upon ligand binding, the receptors dimerize and auto- or trans-phosphorylate specific cytoplasmic tyrosine residues to transmit an intracellular signal that ultimately reaches nuclear transcription factor effectors. Mitogenic signaling by these FGFRs is subsequently mediated via 10 a number of pathways, including the ras/raf/MAP kinase cascade (Ozawa et al., *Teratog. Carcinog. Mutagen.*, 2001, 21, 27-44).

Alternative splicing of the mRNA from the FGFRs 1, 2, and 3 results in a wide range of receptor isoforms with varying ligand-binding properties and specificities. With seven 15 different receptor possibilities and at least 20 ligands in the FGF family, there is a great deal of diversity in the FGF signaling pathway (Powers et al., *Endocr. Relat. Cancer*, 2000, 7, 165-197). Furthermore, expression and localization of the receptor isoforms is regulated in a tissue specific manner. 20 Thus, the various FGFs may exert different influences upon different cell types by interacting with different receptor splice variants to initiate unique intracellular signaling cascades, leading to a panoply of cellular responses (Ozawa et al., *Teratog. Carcinog. Mutagen.*, 2001, 21, 27-44).

25 Fibroblast growth factor receptor 3 (also known FGF receptor-3, FGFR-3, Fgfr3, ACH, JTK4, and CEK2) was cloned from a cDNA library prepared from human chronic myelogenous leukemia (CML) cells and demonstrated to be a biologically active receptor activated by the acidic and basic fibroblast growth 30 factor family members (Keegan et al., *Proc. Natl. Acad. Sci. U. S. A.*, 1991, 88, 1095-1099).

The human fibroblast growth factor receptor 3 gene was mapped to 4p16.3, in a region displaying significant linkage equilibrium to the Huntington's disease (HD) genetic locus 35 located near the terminus of short arm of human chromosome 4. Fibroblast growth factor receptor 3 was found to be expressed in many areas of the brain, including the caudate and putamen (Thompson et al., *Genomics*, 1991, 11, 1133-1142). The mouse

-3-

Fgfr3 gene was mapped to mouse chromosome 5 in a region of synteny with human chromosome 4 (Avraham et al., *Genomics*, 1994, 21, 656-658).

Disclosed and claimed in PCT Publication WO 01/36632 are 5 the isolated nucleotide sequence of two alternatively spliced variants of fibroblast growth factor receptor 3, as well as sequences complementary to these variants. Also claimed are the amino acid sequences of the two fibroblast growth factor receptor 3 variants, expression vectors comprising the nucleic acid sequences encoding the two variants of fibroblast growth factor receptor 3, a host cell transfected by said expression vector, a pharmaceutical composition comprising a pharmaceutically acceptable carrier and as an active ingredient, said expression vector and the amino acid sequence of fibroblast 10 growth factor receptor 3, and a method for detecting a variant nucleic acid sequence comprising a fibroblast growth factor 15 receptor variant (Levine et al., 2001).

Fibroblast growth factor receptor 3 is involved in long bone development and maintenance, and mutations in fibroblast 20 growth factor receptor 3 have been implicated in skeletal malformations. A Lys644Glu point mutation was introduced into the murine fibroblast growth receptor 3 in a knock-in approach, and this mutation resulted in retarded endochondral bone growth, with the severity of the phenotype linked to the copy number of 25 the mutant allele. Molecular analysis revealed that expression of the mutant receptor ultimately caused the activation of cell cycle inhibitors and led to a dramatic expansion of the resting zone of chondrocytes at the expense of the proliferating chondrocytes. The phenotype of these mice strongly resembled 30 those of human patients with achondroplastic syndromes, characterized by dramatically reduced proliferation of growth plate cartilage, macroencephaly and shortening of the long bones. This mouse model confirms an inhibitory role for 35 fibroblast growth factor receptor 3 in bone growth (Li et al., *Hum. Mol. Genet.*, 1999, 8, 35-44).

More than 75 mutations have been recorded to account for seven skeletal syndromes in humans, and the highest rate of germline point mutations in humans occurs in fibroblast growth

-4-

factor receptors 2 and 3. The most common cause for all the mutant phenotypes is gain-of-function by receptor activation through three major mechanisms: receptor dimerization, kinase activation, and increased affinity for the FGF ligands (Kannan and Givol, *IUBMB Life*, 2000, 49, 197-205).

Specifically, disruptions of fibroblast growth factor receptor 3 signaling are associated with multiple forms of skeletal dysplasias, including achondroplastic (ACH) dwarfism and thanatophoric dysplasia, characterized by short limbs, curved bones and neonatal death as well as hypochondroplasia, less severe than ACH, and Crouzon syndrome, characterized by abnormal ossification of cranial sutures (craniosynostosis) (Kannan and Givol, *IUBMB Life*, 2000, 49, 197-205).

Fibroblast growth factor receptor 3 was shown to exert a negative regulatory effect on bone growth and an inhibition of chondrocyte proliferation. Thanatophoric dysplasia is caused by different mutations in fibroblast growth factor receptor 3, and one mutation, TDII FGFR3, has a constitutive tyrosine kinase activity which activates the transcription factor Stat1, leading to expression of the cell-cycle inhibitor p21^{WAF1/CIP1} and growth arrest and abnormal bone development (Su et al., *Nature*, 1997, 386, 288-292).

In contrast to this negative regulation of bone growth, activation of fibroblast growth factor receptor 3 in fibroblasts stimulates proliferation. It appears that fibroblast growth factor receptor 3 signaling can operate along two different pathways, and the Ras-MAPK effector pathway leads to mitogenesis, whereas the STAT1 effector pathway induces cell cycle inhibitors (Kannan and Givol, *IUBMB Life*, 2000, 49, 197-205).

A chromosomal translocation, t(4;14) (p16.3;q32), occurs in 25% of multiple myelomas and lymphoid malignancies, leading to increased expression of fibroblast growth factor receptor 3 and a subset of these tumors also have a mutation which constitutively activates the receptor (Plowright et al., *Blood*, 2000, 95, 992-998; Richelda et al., *Blood*, 1997, 90, 4062-4070). Murine B9 cells transduced with this constitutively activated mutant fibroblast growth factor 3 exhibit enhanced proliferation

-5-

and survival in comparison to controls, indicating an important role for this signaling pathway in tumor development and progression (Plowright et al., *Blood*, 2000, 95, 992-998).

5 The 4p16.3 chromosomal locus has previously been identified as a region of non-random loss of heterozygosity in transitional cell carcinoma. Analysis of a panel of transitional cell carcinomas and cell lines including bladder, renal, and cervical carcinomas showed that, irrespective of whether the tumor has loss of heterozygosity at the 4p16.3 locus, fibroblast growth 10 factor receptor 3 is frequently mutated. Activating mutations in fibroblast growth factor have now been identified in several cancer types, and it seems likely that these mutations contribute to the malignant phenotype (Sibley et al., *Oncogene*, 2001, 20, 686-691).

15 A splice variant of the human fibroblast growth factor receptor 3 mRNA, missing exons 7 and 8 which encode the transmembrane domain but bearing an intact kinase domain, has been reported. The gene product of this variant is predicted to be soluble and intracellular, and immunolocalization studies 20 have shown it to be localized to the nucleus in normal breast epithelial cells and in breast cancer cells, but its role in tumorigenesis is not known (Johnston et al., *J. Biol. Chem.*, 1995, 270, 30643-30650).

Finally, in primary colorectal cancer tissues and cell 25 lines, fibroblast growth factor receptor 3 was found to be frequently inactivated by aberrant splicing and usage of cryptic splice donor sites within exon 7 (Jang et al., *Cancer Res.*, 2000, 60, 4049-4052).

30 The modulation of fibroblast growth factor receptor 3 activity and/or expression is an ideal target for therapeutic intervention aimed at regulating the FGF signaling pathway in the prevention and treatment of many cancers and hyperproliferative diseases.

35 Investigative strategies aimed at studying fibroblast growth factor receptor 3 localization and function have involved the use of specific antibodies directed against a peptide fragment of fibroblast growth factor receptor 3 (Johnston et

-6-

al., *J. Biol. Chem.*, 1995, 270, 30643-30650) and antisense oligonucleotides.

Disclosed and claimed in PCT Publication WO 00/68424 are methods for detecting carcinomas in a biological sample, 5 comprising identifying fibroblast growth factor receptor 3 mutations using nucleic acid or protein sequences, as well as pharmaceutical preparations having an anti-proliferative effect on carcinoma cells comprising an effective amount of agent(s), including antisense oligonucleotides which act by inhibition of 10 wild type or mutant fibroblast growth factor receptor 3 synthesis or expression (Cappellen et al., 2000).

Currently, there are no known therapeutic agents that effectively inhibit the synthesis of fibroblast growth factor receptor 3. Consequently, there remains a long felt need for 15 agents capable of effectively inhibiting fibroblast growth factor receptor 3 function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and therefore may prove to be uniquely useful in a number of therapeutic, 20 diagnostic, and research applications for the modulation of fibroblast growth factor receptor 3 expression.

The present invention provides compositions and methods for modulating fibroblast growth factor receptor 3 expression, including modulation of the truncated mutants and alternatively 25 spliced forms of fibroblast growth factor receptor 3.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, 30 particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding fibroblast growth factor receptor 3, and which modulate the expression of fibroblast growth factor receptor 3. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further 35 provided are methods of modulating the expression of fibroblast growth factor receptor 3 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further

provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of fibroblast growth factor receptor 3 by administering a therapeutically or 5 prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, 10 particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding fibroblast growth factor receptor 3, ultimately modulating the amount of fibroblast growth factor receptor 3 produced. This is accomplished by providing antisense compounds which specifically 15 hybridize with one or more nucleic acids encoding fibroblast growth factor receptor 3. As used herein, the terms "target nucleic acid" and "nucleic acid encoding fibroblast growth factor receptor 3" encompass DNA encoding fibroblast growth factor receptor 3, RNA (including pre-mRNA and mRNA) transcribed 20 from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally 25 referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the 30 RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of fibroblast growth factor receptor 3. In the context of the 35 present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is

-8-

a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding fibroblast growth factor receptor 3. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding fibroblast growth factor receptor 3, regardless of the sequence(s) of such codons.

-9-

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The 5 terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and 10 "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is 15 known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from 20 the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation 25 termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 30 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly 35 translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a

-10-

continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a

5 particular mRNA splice product is implicated in disease.

Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

10 Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

15 In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, 20 refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be 25 complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and 30 "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 35 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function

-11-

of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under 5 physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are 10 identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. 15 Therefore another embodiment of the invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, 20 are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

25 For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of 30 genes expressed within cells and tissues.

Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression 35 as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the

-12-

presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods (reviewed in (To, *Comb. Chem. High Throughput Screen*, **2000**, 3, 235-41)).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed

-13-

of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are 5 often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of 10 antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 15 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external 20 guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar 25 combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate 30 group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' 35 hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the

-14-

oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified 5 backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as 10 sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphoro-15 dithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkyl-20 phosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted 25 polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

30 Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 35 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this

-15-

application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 20 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, 25 certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units 30 are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an 35 oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

-16-

Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds 5 can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular - CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene 10 (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the native 15 phosphodiester backbone is represented as -O-P-O-CH₂-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N- 20 alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are 25 from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, 30 aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar 35 properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes

-17-

2'-dimethylaminoxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., $2'-O-CH_2-O-CH_2-N(CH_3)_2$, also described in examples hereinbelow.

5 A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne (-CH₂-), 10 group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 15 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂), 2'-allyl (2'-CH₂-CH=CH₂), 2'-O-allyl (2'-O-CH₂-CH=CH₂) and 2'-fluoro (2'-F). The 2'- modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar 20 modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States 25 patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, 30 certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or 35 substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and

-18-

natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH₃) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-aminoadenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine

-19-

substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base 5 substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not 10 limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 15 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

20 Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups 25 covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups 30 that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in 35 the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the

-80-

As shown in Table 1, SEQ ID NOS 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 33, 35, 36, 37, 38, 39, 40, 41, 42, 45, 47, 48, 49, 50, 51, 53, 54, 55, 56, 58, 59, 60, 61, 62, 64, 66, 69, 70, 71, 72, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 5 87, 88, 89, 90, 92, 93, 94 and 95 demonstrated at least 55% inhibition of human fibroblast growth factor receptor 3 expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred 10 sites for targeting by compounds of the present invention.

Example 16

15 **Western blot analysis of fibroblast growth factor receptor 3 protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 μ l/well), boiled for 5 minutes and loaded on 20 a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to fibroblast growth factor receptor 3 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands 25 are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

5 1. A compound 8 to 50 nucleobases in length targeted to
a nucleic acid molecule encoding fibroblast growth factor
receptor 3, wherein said compound specifically hybridizes with
said nucleic acid molecule encoding fibroblast growth factor
receptor 3 and inhibits the expression of fibroblast growth
10 factor receptor 3.

2. The compound of claim 1 which is an antisense
oligonucleotide.

3. The compound of claim 2 wherein the antisense
oligonucleotide has a sequence comprising SEQ ID NO: 18, 20, 21,
15 22, 23, 24, 25, 26, 27, 28, 29, 30, 33, 35, 36, 37, 38, 39, 40,
41, 42, 45, 47, 48, 49, 50, 51, 53, 54, 55, 56, 58, 59, 60, 61,
62, 64, 66, 69, 70, 71, 72, 74, 75, 76, 77, 78, 79, 80, 81, 82,
83, 84, 85, 86, 87, 88, 89, 90, 92, 93, 94 or 95.

4. The compound of claim 2 wherein the antisense
20 oligonucleotide comprises at least one modified internucleoside
linkage.

5. The compound of claim 4 wherein the modified
internucleoside linkage is a phosphorothioate linkage.

6. The compound of claim 2 wherein the antisense
25 oligonucleotide comprises at least one modified sugar moiety.

7. The compound of claim 6 wherein the modified sugar
moiety is a 2'-O-methoxyethyl sugar moiety.

8. The compound of claim 2 wherein the antisense
oligonucleotide comprises at least one modified nucleobase.

30 9. The compound of claim 8 wherein the modified
nucleobase is a 5-methylcytosine.

10. The compound of claim 2 wherein the antisense
oligonucleotide is a chimeric oligonucleotide.

35 11. A compound 8 to 50 nucleobases in length which
specifically hybridizes with at least an 8-nucleobase portion of
an active site on a nucleic acid molecule encoding fibroblast
growth factor receptor 3.

12. A composition comprising the compound of claim 1 and

-82-

a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the compound is 5 an antisense oligonucleotide.

15. A method of inhibiting the expression of fibroblast 10 growth factor receptor 3 in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of fibroblast growth factor receptor 3 is inhibited.

16. A method of treating an animal having a disease or 15 condition associated with fibroblast growth factor receptor 3 comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of fibroblast growth factor receptor 3 is inhibited.

17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

18. The method of claim 17 wherein the hyperproliferative 20 disorder is cancer.

19. The method of claim 18 wherein the cancer is colorectal, bladder, bone, lung, cervical, breast or skin.

20. The method of claim 16 wherein the disease or condition is a developmental disorder.